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I, JANENE PEISKER, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. 2004900190 for a patent by CBIO LIMITED as filed on 16 January 2004.

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PROVISIONAL SPECIFICATION

Invention Title: "CHAPERONIN 10 MODULATION OF CYTOKINE AND CHEMOKINE SECRETION"

The invention is described in the following statement:

TITLE

CHAPERONIN 10 MODULATION OF CYTOKINE AND CHEMOKINE SECRETION

FIELD OF INVENTION

This invention relates to a method of modulating immunomodulator secretion in immune cells and treatment of diseases and disorders resulting from excessive immunomodulator secretion. More particularly, this invention relates to a method of modulation of cytokine and chemokine secretion using chaperonin 10 through interaction with Hsp60 protein, Toll-like receptors and/or β1-integrins that mediate cytokine and chemokine secretion.

BACKGROUND OF THE INVENTION

Mammalian chaperonin 10 (also known as heat shock protein 10) and heat shock protein 60 (Hsp60) are mitochondrial proteins involved in protein folding, and are homologues of the bacterial proteins GroES and GroEL, respectively. GroES and chaperonin 10 (Cpn10) oligomerise into seven member rings that bind as a lid onto a cup-like structure comprising seven GroEL or Hsp60 molecules, which tether the denatured proteins (Fiaux *et al.*, 2002, Nature, 418, 207-211; Meyer *et al.*, 2003, Cell, 13, 369-381). Hsp10 and Hsp60 are also frequently found at the cell surface (Belles *et al.*, 1999, Infect Immun, 67, 4191-4200; Feng *et al.*, 2001, Blood, 97, 3505-3512) and in the extracellular fluid (Michael *et al.*, 2003, J Biol Chem, 278, 7607-7616; Johnson *et al.*, 2003, Cir Rev Immunol, 23, 15-44).

HSPs released from dying or otherwise stressed cells are believed to be a source of "danger" signals informing the innate and adaptive immune system of the presence of tissue damage induced by various insults including infection, injury, toxins, heat and/or cellular stress (Johnson *et al.*, 2003, Crit Rev Immunol, 23, 25-44; Wallin *et al.*, 2002, Trends Immunol, 23, 130-135; Beg, 2002, Trends Immunol, 23, 509-512). Human studies have also shown an association between elevated serum Hsp60 and low socioeconomic status, social isolation, psychological distress and increased levels of the pro-inflammatory cytokine, TNF-α (Lewthwaite *et al.*, 2002, Circulation, 106, 196-201).

The Toll-like receptor family plays an important role in inflammation and immunity in insects, animals and plants. Toll-like receptors (TLRs) are expressed on cells of the mononuclear lineage including lymphocytes, macrophages and dendritic cells. TLR activation by pathogens induces intracellular signaling that primarily results in activation of the transcription factor NF-κB (Beg, *supra*), and modulation of cytokine production. However, a series of other pathways can also be triggered, including p38 mitogen activated kinase, c-Jun-N-terminal kinase and extracellular signal related kinase pathways (Flohe *et al.*, *supra*; Triantafilou & Triantafilou, *supra*). The patterns of gene expression induced by ligation of the different TLRs are distinct but often overlap. For instance a large proportion of the genes upregulated by TLR3 agonists and double stranded RNA are also upregulated by TLR4 agonists and LPS (Doyle *et al.*, 2002, Immunity, 17, 251-263). TLR4 activation by LPS in macrophages results in TNF-α, IL-12 IL-1β,

RANTES and MIP1β secretion (Flohe *et al., supra*; Jones *et al.,* 2002, J Leukoc Biol, 69, 1036-1044).

Host Hsp60, Hsp70 and chaperone gp96 molecules are believed to activate the innate and adaptive immune system via TLR2 and TLR4 (Flohe, *et al.*, 2003, J Immunol, 170, 2340-2348; Beg, *supra*; Zanin-Zhorov *et al.,* 2003, Faseb, J17, 1567-1569). TLR2 is also activated by TLT2 agonists, lipoteichoic acid and lipopeptides, which can be components of the outer wall of certain bacteria. TLR4 is also activated by lipoproteins or lipopolysaccharide (LPS) or endotoxin, which is a component of the outer wall of gram-negative bacteria. It is unknown how HSPs interact with and activate TLRs however, it has been reported that HSPs, including Hsp60, can bind to the surface of cells (Beg, *supra;* Habich *et al.*, 2003, FEBS Lett, 533, 105-109). HSPs may form part of a multi-component TLR complex and somehow influence LPS signaling (Triantafilou & Triantafilou, 2002, Trends Immunol, 23, 301-304). It is also possible that HSPs (particularly Hsp60) may play a role in potentiating TLR signaling by low or sub-threshold levels of LPS (Johnson *et al.*, *supra*).

Cpn10 was originally identified as a suppressive factor present early in pregnancy and has shown immunosuppressive activity in experimental autoimmune encephalomyelitis, delayed type hypersensitivity and allograft rejection models (Zhang *et al.,* 2003, J Neurol Sci, 212, 37-46; Morton *et al.,* 2000, Imunol Cell Biol, 78, 603-607). However, the mechanism of action by which Cpn10 exerts its immunosuppressive effects has remained obscure.

SUMMARY OF INVENTION

Surprisingly, the inventors have demonstrated that Cpn10 modulates Toll-like receptor agonist-mediated stimulation of immunomodulator secretion through Toll-like receptors and/or β1-integrins demonstrated by a reduction of TNF-α and RANTES secretion and NF-κB activation. Cpn10 also independently promotes IL-10 production.

The invention is broadly directed to the modulation of Toll-like receptor and/or β1-integrin signaling by chaperonin 10 (Cpn10) to thereby modulate immunomodulator secretion. Although not wishing to be bound by any particular theory, the inventors also propose that Cpn10 inhibition of Toll-like receptor and/or β1-integrin signaling may be mediated by Hsp60.

In a first aspect, the invention provides a method of regulating immunomodulator secretion in an animal, or in one or more cells, or tissues or organs derived therefrom, including the step of administering Cpn10, or a derivative of Cpn10, to the animal, cells, tissues or organs, to thereby modulate Toll-like receptor signaling and thereby regulate immunomodulator secretion.

In a second aspect, there is provided a method of regulating immunomodulator secretion in an animal, or in one or more cells, or tissues or organs derived therefrom including the step of administering Cpn10, or derivative of Cpn10, to the animal, cells, tissues or organs, to thereby modulate Hsp60 protein activity and thereby regulate immunomodulator secretion.

In a third aspect, the invention provides a method of regulating immunomodulator secretion in an animal, or in one or more cells, or tissues or organs derived therefrom, including the step of administering Cpn10, or a

derivative of Cpn10, to the animal, cells, tissues or organs, to thereby modulate β1-integrin signaling and thereby regulate immunomodulator secretion.

According to these aspects, the invention provides a method of modulating immunomodulator secretion to thereby modulate the immune response in an animal in response to acute or chronic inflammatory diseases such as septic shock, inflammatory bowel disease, arthritis, psoriasis, heart disease, atherosclerosis, chronic pulmonary disease, cachexia, multiple sclerosis, GVHD, transplantation and cancer.

Suitably, secretion of the immunomodulator is inducible by a Toll-like receptor agonist.

Preferably, the Toll-like receptor is selected from the group consisting of TLR2, TLR3 and TLR4.

Preferably, the Toll-like receptor agonist is selected from the group consisting of LPS, lipopeptide, double stranded RNA and Hsp60.

Preferably, the lipopeptide is PAM_3CysSK_4.

In one embodiment, where the Toll-like receptor is TLR4, the agonist is LPS and/or Hsp60.

In another embodiment, where the Toll-like receptor is TLR3, the agonist is double stranded RNA.

In yet another embodiment, where the Toll-like receptor is TLR2, the agonist is PAM_3CysSK_4 and/or Hsp60.

Preferably, the cell is an immune cell.

More preferably, the immune cell is a T cell, monocyte, macrophage or a lymphocyte.

Preferably, the animal is a mammal.

More preferably, the mammal is a human.

According to the aforementioned aspects, in one embodiment, the immunomodulator is a pro-inflammatory cytokine, such as TNF-α, or pro-inflammatory chemokine, such as RANTES.

In another embodiment, the immunomodulator is an anti-inflammatory cytokine, such as interleukin-10, or anti-inflammatory chemokine, such as TGF-β.

In embodiments where the immunomodulator is a pro-inflammatory cytokine or chemokine, administration of Cpn10 preferably inhibits, suppresses or otherwise reduces secretion of said immunomodulator.

In embodiments where the immunomodulator is an anti-inflammatory cytokine or chemokine, administration of Cpn10 preferably augments, facilitates or otherwise increases secretion of said immunomodulator.

In a fourth aspect, the invention provides use of Cpn10 to produce or design a modulator which modulates Toll-like receptor signaling and/or Toll-like receptor agonist-inducible immunomodulator secretion.

Preferably, the modulator modulates a Toll-like receptor.

Preferably, the Toll-like receptor is selected from the group consisting of TLR2, TLR3 and TLR4.

More preferably, the Toll-like receptor is TLR4.

In a fifth aspect, the invention contemplates use of Cpn10 to produce or design a modulator of Hsp60 which modulates Hsp60-dependent Toll-like receptor signaling and/or Toll-like receptor agonist-inducible immunomodulator secretion.

Preferably, the modulator modulates the Hsp60-dependent augmentation of Toll-like receptor agonist signaling and immunomodulator secretion.

In one embodiment of this aspect, said Hsp60 modulator can bind to a Hsp60 molecule preventing Hsp60 binding to, interacting with or stimulating a Toll-like receptor or other cell surface receptor(s).

Preferably, said Hsp60 modulator prevents Hsp60 from mediating or contributing to Toll-like receptor signaling.

In another embodiment of this aspect, said Hsp60 modulator can bind to a Toll-like receptor, thereby preventing Hsp60 binding to, interacting with or stimulating said Toll-like receptor.

Preferably, said Hsp60 modulator prevents Hsp60 from mediating or contributing to Toll-like receptor signaling.

In a sixth aspect, the invention provides use of Cpn10 to produce or design a modulator of β1-integrin signaling.

In a seventh aspect, the invention contemplates use of Cpn10 to produce or design a modulator of Hsp60 which modulates Hsp60-dependent β1-integrin signaling and/or β1-integrin agonist-inducible immunomodulator secretion.

Preferably, the modulator modulates the Hsp60-dependent augmentation of β1-integrin agonist signaling and immunomodulator secretion.

In one embodiment of this aspect, said Hsp60 modulator can bind to a Hsp60 molecule preventing Hsp60 binding to, interacting with or stimulating a β1-integrin.

Preferably, said Hsp60 modulator prevents Hsp60 from mediating or

contributing to β1-integrin signaling.

In another embodiment of this aspect, said Hsp60 modulator can bind to a β1-integrin receptor, thereby preventing Hsp60 binding to, interacting with or stimulating said β1-integrin.

Preferably, said Hsp60 modulator prevents Hsp60 from mediating or contributing to β1-integrin signaling.

It will be appreciated, according to the aforementioned aspects, that the modulator may be an agonist, antagonist or blocker.

In an eighth aspect, the invention provides an isolated protein complex comprising Cpn10 and Hsp60.

In one form the isolated protein complex is obtainable from a cell surface or extracellular fluid.

In a ninth aspect, the invention provides an isolated protein complex comprising Cpn10, Hsp60 and a Toll-like receptor.

In a tenth aspect, the invention provides an isolated protein complex comprising Cpn10, Hsp60 and a β1-integrin.

In an eleventh aspect, the invention provides an isolated protein complex comprising Cpn10, Hsp60, a β1-integrin and a Toll-like receptor.

In one embodiment of the eighth to eleventh aspects, the invention provides use of the isolated protein complex to produce or design an agent capable of regulating immunomodulator secretion.

In one particular embodiment said agent suppresses, reduces or otherwise inhibits pro-inflammatory cytokine or chemokine secretion normally induced by a Toll-like receptor agonist.

In another embodiment said agent augments, facilitates or otherwise increases anti-inflammatory cytokine or chemokine secretion normally induced by a Toll-like receptor agonist.

In a twelfth aspect, the invention provides a pharmaceutical composition, when used according to any one of the first to third aspects, comprising Cpn10 and a pharmaceutically acceptable carrier, diluent or excipient.

Throughout this specification, "comprise", "comprises" and "comprising" are used inclusively rather than exclusively, will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1: Cpn10 inhibits LPS-induced activation of RAW264.7 cells and pro-inflammatory mediator production.

(A) Cpn10-mediated inhibition of LPS-induced NF-κB activity. In 9 separate experiments 100 υg/ml of Cpn10 (Cpn10 +) or buffer (Cpn10 -) was preincubated with RAW264-HIV-LTR-LUC cells for 2 h. LPS was then added at 5, 1 and 0.2 ng/ml and luciferase activity measured 2 h later. The relative light units (RLU) of luciferase obtained for 5 ng/ml of LPS was set at 100% relative luciferase activity, and 0% represents the RLU obtained in the absence of LPS. Cpn10 alone did not stimulate significant RLU (data not shown). The mean percentage reduction (± SD) in RLU mediated by Cpn10 is indicated for each concentration of LPS, and the significance calculated using a paired t test.

(B) Cpn10-mediated inhibition of LPS-induced TNF-α secretion. RAW264.7 cells were incubated with 0.5 ng/ml of LPS in the presence of 20 υg/ml of Cpn10 (Cpn10 +) or buffer (Cpn10 -) and after 4 hours the supernatant was analysed for TNF-α by ELISA; (eight separate experiments are shown). The mean percentage reduction (± SD) in TNF-α secretion and the significance, calculated using a paired t test, is indicated.

(C) Cpn10-mediated inhibition of LPS-induced RANTES secretion. RAW264.7 cells were incubated with 5 ng/ml of LPS in the presence of 100 υg/ml of Cpn10 and after 4 hours the supernatant was analysed for RANTES by ELISA; (seven separate experiments are shown). The percentage reduction and significance were calculated as for B.

FIG. 2 Effect of Cpn10 on cytokine secretion in murine systems.

(A) Cpn10 treatment reduced capacity of LPS-stimulated peritoneal macrophages to produce TNF-α. C57BL/6 mice (n=3) were treated with Cpn10 (Cpn10 +) or control diluent (Cpn10 -). Peritoneal macrophages were harvested by peritoneal lavage on day 6 and pooled from individual animals within the treatment group. Cells were plated at $2x10^5$/well in the presence of LPS (1 υg/ml). Culture supernatants were collected at 5 hours and levels of TNF-α were assessed by ELISA. (Wells without LPS produced no detectable TNF-α - data not shown). Mean ± SE of triplicate wells are shown, and are normalized to production per 10^5 macrophages based on CD11b staining. Data from two identical experiments are shown and the average percentage reduction is indicated with the significance calculated by ANOVA.

(B) Cpn10 treatment augmented IL-10 production from splenocytes. C57BL/6 mice were treated with either Cpn10 or control diluent as above. Splenocytes were harvested on day 6 and pooled from individual animals within a treatment group and cultured at $5x10^5$/well in the presence of LPS (10 ug/ml). Culture supernatants were collected at 48 hours and levels of IL-10 determined by ELISA. Means ± SE of triplicate wells are shown. Average percentage increase is shown with statistics calculated as for A.

(C) Cpn10 treatment reduced TNF-α production from $IL10^{-/-}$ peritoneal macrophages. $IL10^{-/-}$ C57BL/6 mice were treated with Cpn10 or control diluent as above and peritoneal macrophages were harvested as for A. After 5 hours of culture in the presence of LPS (0.1, 1 or 10 ug/ml) TNF-α was determined in culture supernatants by ELISA. Mean ± SE of triplicate wells for one representative experiment is shown. TNF-α levels were compared for Cpn10 treated and control animals using a non-parametric t test.

FIG. 3 Cpn10-treatment of human peripheral blood mononuclear cells (PBMC) reduces LPS-induced TNF-α secretion.

(A) The LPS-induced TNF-α levels secreted by PBMC cultures were determined in 10 separate experiments using PBMC from 7 different donors. Cpn10 (400 ug/ml) was added to the PBMC 2 hours prior to the addition of LPS. The percentage reduction and significance were calculated as for Fig. 2B.

(B, C) Dose-dependent Cpn10-mediated reduction in TNF-α and increase in IL-10 secretion after LPS stimulation. A representative experiment

using PBMC from a single donor is shown using a concentration range of Cpn10 and LPS. Supernatants were removed after 4 hrs and analysed for TNF-α (B) and IL-10 (C).

FIG. 4 Cpn10 activity in murine inflammatory models.

(A) Cpn10 reduces LPS-induced serum TNF-α and RANTES levels and increases IL-10 levels. In 5 separate experiments C57BL/6 mice (n=3 or 4 per group) were given buffer (Cpn10 -) or 100 υg of Cpn10 (Cpn10 +) iv 30 mins before iv administration of 10 υg of LPS. After 1.5 hours the animals were sacrificed and serum TNF-α, RANTES and IL-10 levels determined; (the latter two were assessed in 3/5 experiments). Error bars represent standard errors within each experiment. The percentage reduction in TNF-α and RANTES and increase in IL-10 (± SD) is indicated and the significance calculated using ANOVA tests.

(B) Pre-transplant treatment with Cpn10 delays GVHD mortality and reduces clinical severity of acute disease. Syngeneic negative controls (n=8) (white circles) represent B6D2F1 mice transplanted with syngeneic B6D2F1 bone marrow and T cells. Allogeneic positive controls (n=10) (white squares) represent diluent pre-treated B6D2F1 recipient mice transplanted with cells from diluent pre-treated B6 donor mice. Allogeneic + Cpn10 (n=10) (black squares) represent B6D2F1 recipients receiving bone marrow and T cell grafts from B6 donor mice where both recipients and donors were pre-treated with Cpn10 prior to transplantation. Kaplan-Meier survival curves and clinical scores are shown for the three groups and the allogeneic groups treated with and without Cpn10

compared by Log Rank Statistic and non-parametric t test, respectively. Clinical scores were only significantly different on day 7.

FIG. 5 Cpn10 activity is blocked by anti-Hsp60 antiserum. RAW264-HIV-LTR-LUC cells were treated with anti-Hsp60 or control serum and Cpn10 (100 ng/ml) (Cpn10 +) or buffer (Cpn10 -) added for a further 2 hours. LPS was then added and luciferase activity measured 2 hours later. The mean percentage reduction (± SD) in Relative light units for 5, 1 and 0.2 ng/ml LPS taken together is indicated and the significance calculated using a paired t test.

FIG. 6 (A) Area of subcutaneous granuloma present in BALB/c mice given CFA with and without Cpn10 treatment.

(B-E) PAM3CysSK$_4$-induced macrophage activation is inhibited by Cpn10 in Raw 264 -HIV LTR Luc cells. (B) Cpn10 or diluent added for 2 hrs followed by addition of PAM3CysSK$_4$ for 2 h followed by the luciferase assay. (C) Cpn10 or diluent added for 2 hrs, followed by washing to remove Cpn10 or diluent, followed by addition of PAM3CysSK$_4$ for 2 h and then the luciferase assay. (D) Cpn10 or diluent added for 2 hrs followed by addition of PAM3CysSK$_4$ for 2 h and then the luciferase assay. (E) The same protocol was used in (B) except the cells were activated with LPS instead of PAM3CysSK$_4$. The top panel shows the relative light unit data, and the bottom the percent inhibition mediated by Cpn10.

FIG. 7 Inhibition of polyIC/TLR3 mediated HIV LTR activation compared with inhibition of LPS/TLR4 inhibition by Cpn10. Note the % inhibition of RLU without Cpn10 were set to zero.

FIG. 8 Mean (± SEM) weight loss during adjuvant arthritis (n=10 per group).

FIG. 9 Cpn10 administered to healthy volunteers reduced the LPS-driven TNF-α response of PBMC *in vitro*. Subjects were infused with placebo (n=3), or 1 mg (n=1), 2.5 mg (n=3), 5 mg (n=3) Cpn10. PBMC isolated 12 hours before (pre) and 8 hours after infusion (post) were stimulated with a range of LPS concentrations *in vitro*.

(A) Data represent the difference between the LPS-driven TNF-α production from cells isolated post- vs. pre- Cpn10 infusion.

(B) TNF-α levels at pre- and post-treatment.

DETAILED DESCRIPTION OF INVENTION

The present inventors discovered that, in a number of different human and murine *in vitro* and *in vivo* systems, Cpn10 inhibits LPS-mediated secretion of the pro-inflammatory cytokine TNF-α and the pro-inflammatory chemokine RANTES, and increases LPS-induced secretion of the anti-inflammatory cytokine IL-10 in monocytes, macrophages and lymphocytes.

The inventors have unexpectedly demonstrated that Cpn10 modulates immunomodulator secretion by modulating intracellular signaling by TLR4, TLR3 and TLR2 agonists. The inventors believe Cpn10 binds Hsp60, preventing Hsp60 interacting with, or contributing to TLR4, TLR3 and TLR2 signaling, and thereby inhibiting Hsp60-mediated augmentation of TLR signaling.

Hsp60 is also reported to bind to β1 integrin (Iwata *et al.*, 2000, J Dermatol Phy, 23,75-86). β1 integrin is capable of mediating signal transduction and β1 integrin-activation can alter the behavior of cells in response to inflammatory stimuli (Zanin-Zhorov, *supra*). Thus Hsp60-mediated signaling via β1 integrin may augment TLR-mediated signaling. The inventors believe Cpn10 may also inhibit β1 integrin augmentation of TLR-mediated signaling by binding to Hsp60.

Cpn10 reduces LPS-stimulated NFκB activation, and TNF-α and RANTES secretion, and increases IL-10 secretion in a dose-dependent manner in human and mouse cell lines and freshly isolated cells *in vitro*.

Cpn10 reduces TNF-α and RANTES production and increases IL-10 production in an *in vivo* non-lethal endotoxemia model in mice. Cpn10 also has significant immunosuppressive activity in an *in vivo* mouse transplantation model and Cpn10 treatment increases the survival rate of mice suffering from graft versus host disease.

Cpn10 also reduces cachexia in rats suffering from adjuvant-induced arthritis. Elevated levels of inflammatory cytokines are associated with cachexia in a number of diseases, such as cancer and rheumatoid arthritis.

In addition Cpn10 improves wound healing in an *in vivo* mouse model.

The invention also shows that Cpn10 administered as a single intravenous dose to humans *in vivo*, markedly reduces the pro-inflammatory cytokine response following LPS-stimulation *ex vivo* in a dose-responsive manner clearly demonstrating that Cpn10 has immunomodulatory effects in human clinical trial subjects.

Excessive inflammation or uncurtailed immune responses are detrimental to a host, hence a number of negative feedback systems have evolved to dampen production of pro-inflammatory mediators. One such negative feedback mechanism includes IL-10, an important immunoregulatory cytokine secreted by mononuclear cells and monocytes, which is involved in limitation of inflammatory responses and induction of immune tolerance.

Cpn10 inhibits but does not abolish TNF-α and RANTES secretion. This is a desirable characteristic since complete TNF-α removal (for example, by anti-TNF-α antibodies) can result in compromised immunity, predisposing patients to infection, and reduced tumour surveillance, predisposing patients to increased frequency of tumours.

The ability of Cpn10 to reduce secretion of immunomodulators indicates that Cpn10 will find therapeutic application in conditions where excessive pro-inflammatory immunomodulator secretion leads to disease.

Many diseases are associated with excessive or chronic inflammation, hence modulation of TLR receptor signaling resulting in modulation of cytokine secretion may have widespread clinical benefits. For example, excessive secretion of pro-inflammatory cytokines, such as TNF-α is one of the leading causes of death in acute conditions such as septic shock, and it is one of the main factors contributing to ongoing tissue damage in chronic inflammatory diseases such as inflammatory bowel disease (IBD), arthritis, psoriasis, congestive heart disease, multiple sclerosis, and chronic obstructive pulmonary disease.

In tissue or organ transplantation the host or donor lymphocytes can recognise the donor or host cell antigens, respectively, as foreign and release cytokines which activate cells of the innate immune system resulting in rejection of the transplanted tissue or organ or graft versus host disease. Immunosuppressive drugs play a large role in the therapeutic treatment and management of transplant rejection and graft versus host disease. However, the drugs evoke severe side effects in patients, they are very expensive and in some patients they are poorly effective.

For the purposes of the invention, by "immunomodulator" is meant a molecular mediator secreted by cells of the immune system or a molecular mediator which interacts with cells of the immune system that plays a role in activation, maintenance, maturation, inhibition, suppression or augmentation of an immune response.

By "*cytokine*" is meant a molecular mediator secreted by cells of the immune system that plays a role in activation, maintenance, maturation, inhibition, suppression or augmentation of an immune response. Non-limiting examples of cytokines are TNF-α, interleukin-1 and interleukin-10.

By "*chemokine*" is meant a molecular mediator that acts to promote and/or regulate cell migration and activation. Non-limiting examples of a chemokine is RANTES.

By "*pro-inflammatory immunomodulator*" is meant a cytokine or chemokine that plays a role or has some involvement in an inflammatory process or inflammatory response.

By "*anti-inflammatory immunomodulator*" is meant a cytokine or chemokine that plays a role in inhibiting, suppressing or otherwise decreasing an inflammatory response.

By "*immunosuppressive agent*" is meant an agent that can prophylactically or therapeutically suppress an immune response or an autoimmune response, for example, against a transplanted allogeneic or xenogeneic cell, tissue or organ.

By "*isolated*" is meant material that has been removed from its natural state or otherwise been subjected to human manipulation. Isolated material may be substantially or essentially free from components that normally accompany it in its natural state, or may be manipulated so as to be in an artificial state together with components that normally accompany it in its natural state. Isolated material may be in native, chemical synthetic or recombinant form.

By "*protein*" is meant an amino acid polymer. The amino acids may be natural or non-natural amino acids D- and L- amino acids, as are well understood in the art.

A "*peptide*" is a protein having no more than fifty (50) amino acids.

A "*polypeptide*" is a protein having more than fifty (50) amino acids.

The term "*nucleic acid*" as used herein designates single-or double-stranded mRNA, RNA, cRNA, RNAi and DNA inclusive of cDNA and genomic DNA.

Cpn10, Cpn10 derivatives and Cpn10 mimetics

"*Cpn10*" or "*chaperonin 10*" refers to Cpn10 proteins inclusive of derivatives, fragments and variants thereof. A Cpn10 protein may comprise

naturally occurring modification such as glycosylation or acetylation. Cpn10 may be derived from any species, including human, mouse, rat and others. Cpn10 may be recombinant or native and may comprise natural and/or non-natural D- or L-amino acids as are well understood in the art.

In one embodiment, a "*fragment*" includes an amino acid sequence that constitutes less than 100%, but at least 20%, preferably at least 30%, more preferably at least 80% or even more preferably at least 90% of said polypeptide.

The fragment includes a "*biologically active fragment*", which retains biological activity of a given protein or peptide. For example, a biologically active fragment of Cpn10 capable of inducing immunosuppression in a subject may be used in accordance with the invention. The biologically active fragment constitutes at least greater than 1% of the biological activity of the entire polypeptide or peptide, preferably at least greater than 10% biological activity, more preferably at least greater than 25% biological activity and even more preferably at least greater than 50% biological activity.

As used herein, *"variant"* proteins are proteins in which one or more amino acids have been replaced by different amino acids. Protein variants of Cpn10 that retain biological activity of native or wild type Cpn10 may be used in accordance with the invention. It is well understood in the art that some amino acids may be changed to others with broadly similar properties without changing the nature of the activity of the polypeptide (conservative substitutions). Generally, the substitutions which are likely to produce the greatest changes in a polypeptide's properties are those in which (a) a hydrophilic residue (*e.g.*, Ser or Thr) is substituted for, or by, a hydrophobic residue (*e.g.* Leu, Ile, Phe or Val); (b)

a cysteine or proline is substituted for, or by, any other residue; (c) a residue having an electropositive side chain (*e.g.*, Arg, His or Lys) is substituted for, or by, an electronegative residue (*e.g.*, Glu or Asp) or (d) a residue having a bulky side chain (*e.g.*, Phe or Trp) is substituted for, or by, one having a smaller side chain (*e.g.*, Ala, Ser) or no side chain (*e.g.*, Gly).

With regard to protein variants, these can be created by mutagenising a polypeptide or by mutagenising an encoding nucleic acid, such as by random mutagenesis or site-directed mutagenesis. Examples of nucleic acid mutagenesis methods are provided in Chapter 9 of CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Ausubel *et al.*, *supra* which is incorporated herein by reference.

As used herein, "*derivative*" proteins of the invention are proteins, such as Cpn10 proteins, which have been altered, for example by conjugation or complexing with other chemical moieties or by post-translational modification techniques as would be understood in the art, inclusive of fusion partner proteins.

Other derivatives contemplated by the invention include, but are not limited to, pegylation, modification to side chains, incorporation of unnatural amino acids and/or their derivatives during peptide, polypeptide or protein synthesis and the use of crosslinkers and other methods which impose conformational constraints on the polypeptides, fragments and variants of the invention. Examples of side chain modifications contemplated by the present invention include modifications of amino groups such as by acylation with acetic anhydride; acylation of amino groups with succinic anhydride and tetrahydrophthalic anhydride; amidination with methylacetimidate;

carbamoylation of amino groups with cyanate; pyridoxylation of lysine with pyridoxal-5-phosphate followed by reduction with $NaBH_4$; reductive alkylation by reaction with an aldehyde followed by reduction with $NaBH_4$; and trinitrobenzylation of amino groups with 2, 4, 6-trinitrobenzene sulphonic acid (TNBS).

The carboxyl group may be modified by carbodimide activation via O-acylisourea formation followed by subsequent derivitization, by way of example, to a corresponding amide.

The guanidine group of arginine residues may be modified by formation of heterocyclic condensation products with reagents such as 2,3-butanedione, phenylglyoxal and glyoxal.

Sulphydryl groups may be modified by methods such as performic acid oxidation to cysteic acid; formation of mercurial derivatives using 4-chloromercuriphenylsulphonic acid, 4-chloromercuribenzoate; 2-chloromercuri-4-nitrophenol, phenylmercury chloride, and other mercurials; formation of a mixed disulphides with other thiol compounds; reaction with maleimide, maleic anhydride or other substituted maleimide; carboxymethylation with iodoacetic acid or iodoacetamide; and carbamoylation with cyanate at alkaline pH.

Tryptophan residues may be modified, for example, by alkylation of the indole ring with 2-hydroxy-5-nitrobenzyl bromide or sulphonyl halides or by oxidation with N-bromosuccinimide.

Tyrosine residues may be modified by nitration with tetranitromethane to form a 3-nitrotyrosine derivative.

The imidazole ring of a histidine residue may be modified by N-carbethoxylation with diethylpyrocarbonate or by alkylation with iodoacetic acid derivatives.

Examples of incorporating unnatural amino acids and derivatives during peptide synthesis include but are not limited to, use of 4-amino butyric acid, 6-aminohexanoic acid, 4-amino-3-hydroxy-5-phenylpentanoic acid, 4-amino-3-hydroxy-6-methylheptanoic acid, t-butylglycine, norleucine, norvaline, phenylglycine, ornithine, sarcosine, 2-thienyl alanine and/or D-isomers of amino acids.

Derivatives may also include fusion partners and epitope tags. Well known examples of fusion partners include, but are not limited to, glutathione-S-transferase (GST), Fc portion of human IgG, maltose binding protein (MBP) and hexahistidine (HIS_6), which are particularly useful for isolation of the fusion protein by affinity chromatography. For the purposes of fusion polypeptide purification by affinity chromatography, relevant matrices for affinity chromatography are glutathione-, amylose-, and nickel- or cobalt-conjugated resins respectively. Many such matrices are available in "kit" form, such as the QIAexpress™ system (Qiagen) useful with (HIS_6) fusion partners and the Pharmacia GST purification system.

One particular example of a fusion partner is GST, such as described in Ryan *et al.* (1995, J. Biol. Chem., 270, 22037-22043). Upon cleavage of GST-Cpn10 the derivative GSM-Cpn10 protein is produced, for example. In some cases, the fusion partners also have protease cleavage sites, such as for Factor X_a or Thrombin, which allow the relevant protease to partially digest the fusion

polypeptide of the invention and thereby liberate the recombinant polypeptide of the invention therefrom. The liberated polypeptide can then be isolated from the fusion partner by subsequent chromatographic separation.

Fusion partners according to the invention also include within their scope "*epitope tags*", which are usually short peptide sequences for which a specific antibody is available. Well known examples of epitope tags for which specific monoclonal antibodies are readily available include c-myc, haemagglutinin and FLAG tags.

Cpn10 proteins of the invention (inclusive of fragments, variants, derivatives and homologues) may be prepared by any suitable procedure known to those of skill in the art, including chemical synthesis and recombinant expression.

Preferably, Cpn10 is recombinant Cpn10.

For example, the recombinant Cpn10 protein may be prepared by a procedure including the steps of:

(i) preparing an expression construct which comprises an isolated nucleic acid encoding Cpn10, operably-linked to one or more regulatory nucleotide sequences in an expression vector;

(ii) transfecting or transforming a suitable host cell with the expression construct; and

(iii) expressing the recombinant protein in said host cell.

Modulators of cytokine secretion

The present invention provides methods of modulating secretion of immunomodulator, such as pro-inflammatory and anti-inflammatory cytokine and

chemokines, by inhibiting, suppressing or otherwise reducing Toll-like and/or β1-integrin signaling. It will also be appreciated that immunomodulator secretion can be modulated by augmenting, strengthening or increasing Toll-like and/or β1-integrin signaling.

The inventors demonstrate that Cpn10 exerts its immunosuppressive effects on immune cells through inhibition of Toll-like and/or β1-integrin signaling. It is also proposed that the Cpn10 inhibition of Toll-like and/or β1-integrin signaling may be mediated by Hsp60.

Therefore the present invention contemplates use of Cpn10, a protein complex comprising Cpn10 and Hsp60, a protein complex comprising Cpn10, Hsp60 and a Toll-like receptor, and a protein complex comprising Cpn10, Hsp60 and a β1-integrin to produce or design a modulator of immunomodulator secretion.

The modulators of immunomodulator secretion could be agonists, antagonists or mimetics of Cpn10, a protein complex comprising Cpn10 and Hsp60, a protein complex comprising Cpn10, Hsp60 and a Toll-like receptor, and a protein complex comprising Cpn10, Hsp60 and a β1-integrin.

The term *"mimetic"* is used herein to refer to molecules that resemble particular structural and/or functional regions or domains of proteins or peptides, and includes within its scope the terms *"agonist"*, *"analogue"* and *"antagonist"* as are well understood in the art.

An *"agonist"* refers to a molecule, such as a drug, enzyme activator or protein, which enhances activity of another molecule or receptor site.

For example, modulators of immunomodulator secretion may be identified by way of screening libraries of molecules such as synthetic chemical libraries, including combinatorial libraries, by methods such as described in Nestler & Liu, 1998, Comb. Chem. High Throughput Screen. 1, 113 and Kirkpatrick *et al.*, 1999, Comb. Chem. High Throughput Screen 2, 211.

It is also contemplated that libraries of naturally-occurring molecules may be screened by methodology such as reviewed in Kolb, 1998, Prog. Drug. Res. 51, 185.

More rational approaches to designing modulators as described herein may employ computer assisted screening of structural databases, computer-assisted modelling and/or design, or more traditional biophysical techniques which detect molecular binding interactions, as are well known in the art.

Computer-assisted structural database searching, modelling and design is becoming increasingly utilized as a procedure for engineering agonists and antagonist molecules. Examples of database searching methods may be found in International Publication WO 94/18232 (directed to producing HIV antigen mimetics), United States Patent No. 5,752,019 and International Publication WO 97/41526 (directed to identifying EPO mimetics), each of which is incorporated herein by reference.

Generally, other applicable methods include any of a variety of biophysical techniques which identify molecular interactions. Methods applicable to potentially useful techniques such as competitive radioligand binding assays, analytical ultracentrifugation, microcalorimetry, surface plasmon resonance and optical biosensor-based methods are provided in Chapter 20 of CURRENT

PROTOCOLS IN PROTEIN SCIENCE Eds. Coligan *et al.*, (John Wiley & Sons, 1997) which is incorporated herein by reference.

Pharmaceutical compositions

The invention provides a use of Cpn10 for targeting Toll-like and/or β1-integrin and Hsp60-mediated immunomodulator secretion. Thus, the present invention contemplates use of Cpn10 for the therapeutic or prophylactic treatment of diseases, disorders or medical conditions caused by abnormal, excessive or inappropriate cytokine secretion and cell, tissue or organ transplantation, such as septic shock, inflammatory bowel disease, arthritis, psoriasis, congestive heart disease, cachexia, cancer, GVHD and chronic pulmonary disease. The present invention also contemplates use of Cpn10 for the treatment of diseases responsive to inhibition of pro-inflammatory cytokine secretion.

The invention also provides pharmaceutical compositions that comprise Cpn10 or a derivative of Cpn10.

Suitably, the pharmaceutical composition comprises an appropriate pharmaceutically-acceptable carrier, diluent or excipient.

By "*pharmaceutically-acceptable carrier, diluent or excipient*" is meant a solid or liquid filler, diluent or encapsulating substance that may be safely used in systemic administration. Depending upon the particular route of administration, a variety of carriers, well known in the art may be used. These carriers may be selected from a group including sugars, starches, cellulose and its derivatives, malt, gelatine, talc, calcium sulfate, vegetable oils, synthetic oils, polyols, alginic acid, phosphate buffered solutions, emulsifiers, isotonic saline and salts such as

mineral acid salts including hydrochlorides, bromides and sulfates, organic acids such as acetates, propionates and malonates and pyrogen-free water.

A useful reference describing pharmaceutically acceptable carriers, diluents and excipients is Remington's Pharmaceutical Sciences (Mack Publishing Co. N.J. USA, 1991) which is incorporated herein by reference.

Any safe route of administration may be employed for providing a patient with the composition of the invention. For example, oral, rectal, parenteral, sublingual, buccal, intravenous, intra-articular, intra-muscular, intra-dermal, subcutaneous, inhalational, intraocular, intraperitoneal, intracerebroventricular, transdermal and the like may be employed. Intra-muscular and subcutaneous injection is appropriate, for example, for administration of immunogenic compositions, vaccines and DNA vaccines.

Dosage forms include tablets, dispersions, suspensions, injections, solutions, syrups, troches, capsules, suppositories, aerosols, transdermal patches and the like. These dosage forms may also include injecting or implanting controlled releasing devices designed specifically for this purpose or other forms of implants modified to act additionally in this fashion. Controlled release of the therapeutic agent may be effected by coating the same, for example, with hydrophobic polymers including acrylic resins, waxes, higher aliphatic alcohols, polylactic and polyglycolic acids and certain cellulose derivatives such as hydroxypropylmethyl cellulose. In addition, the controlled release may be effected by using other polymer matrices, liposomes and/or microspheres.

The above compositions may be administered in a manner compatible with the dosage formulation, and in such amount as is pharmaceutically-effective.

The dose administered to a patient, in the context of the present invention, should be sufficient to affect a beneficial response in a patient over an appropriate period of time. The quantity of agent(s) to be administered may depend on the subject to be treated inclusive of the age, sex, weight and general health condition thereof, factors that will depend on the judgement of the practitioner.

So that the present invention may be more readily understood and put into practical effect, the skilled person is referred to the following non-limiting examples.

EXAMPLES

Example 1 - Materials and Methods

Production and Purification of Cpn10

Frozen recombinant Cpn10 was dissolved in 50 mM Tris buffer pH 7.6, 150 mM NaCl and was stored at -20^{0}C. The purity of Cpn10 was determined to be >97%. Aliquots were thawed once prior to use. All batches of Cpn10 were active in a rhodanese refolding assay and showed the same molar activity as GroES (data not shown).

LPS and endotoxin assays

LPS from *E. coli* (Sigma L-6529. Strain 055:B5) was dissolved in distilled water and stored at 4^{0}C at 1 mg/ml in glass vials. Immediately prior to use the solution was vigorous vortexed for 5 mins before aliquots were taken. In medium 1 ng/ml of LPS corresponded to 0.57 endotoxin units (EU)/ml. LPS contamination of Cpn10 was determined by the Limulus Amebocyte Lysate assay (BioWhitiker) and all batches of Cpn10 contained <1 EU/mg of purified Cpn10 protein.

Cell lines

K562 (human erythroleukemia), Mono Mac 6 (human monocytic line), U937 (human histiocytic lymphoma), P815 (mouse mastocytoma), EL4 (mouse T cell lymphoma), Jurkat (human T cell leukemia), RAW 264.7 (ATCC TIB 71, mouse macrophage), L929 (mouse fibrosarcoma), B16 (mouse melanoma), HeLa (human cervical carcinoma), and MCA-2 (mouse fibrosarcoma) cell lines were shown to be mycoplasma negative. Cells were grown in medium comprising RPMI 1640 (Gibco Labs, Life Technologies, Grand Island, N.Y., USA), 10% fetal calf serum (Life Technologies), 2 mM glutamine (Sigma), 10 mM HEPES (Sigma), 100 υg/ml of streptomycin and 100 IU/ml of penicillin (CSL Ltd, Melbourne, Australia). The culture medium was determined to have endotoxin levels < 0.01 ng/ml.

RAW264-HIV-LTR-LUC bioassay

RAW264-HIV-LTR-LUC cells were cultured in the presence of G418 (200 υg/ml) for a week after recovery from liquid nitrogen and grown as suspension cultures (Greiner labortechnik, Frickenhausen, Germany). RAW264-HIV-LTR-LUC cells were disaggregated by repeated pipetting and plated at 2.5 x 10^5 cells/ 24 well and incubated overnight (37^0C and 5% CO_2). Cpn10 was then added for 2 h followed by LPS at the indicated concentrations, and after a further 2 h the adherent cells were processed for the luciferase assay (Luciferase Assay System, Promega. Luciferase activity was read for 15 sec on a Turner Designs Luminometer TD 20/20.

RAW264.7 TNF-α and RANTES assays

Cpn10 and LPS at the indicated concentrations were added to RAW264.7 cells that had been seeded at 5 x 10^4 cells/96 well and cultured overnight. After 3 h (RANTES assay) or 2 hours (TNF-α assay) supernatants (150 µl) were collected and analysed in triplicate for RANTES and TNF-α by Duoset ELISA kit (R & D Systems). The optical density (450 nm) of each sample was determined using a microplate reader (Magellan 3, Sunrise - Tecan, Durham, NC).

Cytokine production from splenocytes and macrophages derived from Cpn10 treated animals

C57BL/6 IL-$10^{-/-}$ mice (H-2^b, Ly-5.2^+) were supplied by the Australian National University (Canberra, Australia). The culture medium used throughout was 10% FCS/IMDM (JRH Biosciences, Lenexa, KS), supplemented with 50 units/ml penicillin, 50 µg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acid, 0.02 mM ß-mercaptoethanol, and 10 mM HEPES, and cells were cultured at pH 7.75, 37°C and 5% CO_2. C57BL/6 mice (n=3 per group) were treated with subcutaneous injections of Cpn10 (100 ug) or diluent daily for 5 days, peritoneal macrophages were harvested the next day by peritoneal lavage and pooled from individual animals within the treatment group. Cells were plated in triplicate at 2x10^5/well in the presence of LPS (1 ug/ml). Culture supernatants were collected at 5 h and levels of TNF-α were assessed by ELISA (see below). Results were normalized to production per 10^5 macrophages based on CD11b staining by FACS analysis of input cells. For IL-10 determination splenocytes were harvested from the same animals and pooled as above and cultured in triplicate at 5 x 10^5/well in the absence (not shown) or

presence of LPS (10 ug/ml). Culture supernatants were collected at 48 h and levels of IL-10 determined by ELISA (see below).

Cytokine assays for murine cells stimulated in vitro with LPS

The monoclonal antibody pairs used in the TNF-α and IL-10 ELISA assays were purchased from PharMingen (San Diego, CA) and used at concentrations recommended by the manufacturer. Supernatants were diluted in culture medium 1:1 IL-10 and TNF-α. Cytokines were captured by the capture antibody, and detected by the direct biotin-labelled detection antibody. Strepavidin-labelled horse-radish peroxidase (Kirkegaard and Perry laboratories, Gaithersburg, MD) and substrate (Sigmafast OPD) was then used to measure immobilised biotin. Plates were read at 492 nm using the Spectraflour Plus microplate reader (Tecan). Recombinant cytokines (PharMingen) were used as standards for ELISA assays. Standards were run in duplicate and the sensitivity of the assays was 15 pg/ml for IL-10 and TNF-α.

Human PBMC TNF-α and IL-10 assays

Human peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood from healthy volunteers by buoyant density gradient centrifugation on Ficoll-Hypaque. PBMCs (1.25×10^6 cells/ml) were dispensed at 800 µl/ 24 well (Greiner). Cpn10 was then added for 5 h, followed by LPS and after 20 h, supernatants were collected and duplicate samples analysed for TNF-α and IL-10 production with a human TNF-α IL-10 Duoset ELISA kit (R & D Systems).

Mouse serum TNF-α, RANTES and IL-10 assays after LPS injection

Female 8-10 week old BALB/c mice (Animal Resource Centre, Perth, Australia) were placed under a heating lamp for approximately 10 minutes, then restrained and Cpn10 injected i.v. at the specified doses. After 30 mins, 10 μg LPS was injected i.v. using the same protocol. At 1.5 hours post LPS injection, blood was collected by heart puncture into 1 ml clotting accelerator tubes (MiniCollect, Interpath) and stored at 4°C for analysis of serum TNF-α and RANTES using the ELISA kits (R & D Systems). IL-10 production in serum was measured with mouse OptEIA IL-10 specific ELISA (BD Biosciences Pharmingen).

Bone marrow transplantation and graft versus host disease (GVHD)

Female 8 -14 week old C57BL/6 (B6, H-2^b, Ly-5.2^+), B6 $Ptprc^a$ $Ly\text{-}5^a$ (H-2^b, Ly-5.1^+) and B6D2F1 (H-$2^{b/d}$, Ly-5.2^+) mice were purchased from the Australian Research Centre (Perth, Western Australia, Australia). Cpn10 (100 ug per animal) or control diluent was injected subcutaneously daily for 5 days into donor and recipient animals prior to transplant. Mice were housed in sterilised microisolator cages and received acidified autoclaved water (pH 2.5) and normal food for the first two weeks post-transplantation. Mice were transplanted according to a standard protocol described previously (Hill *et al.*, 1997, Blood, 90, 3204-3213; Hill *et al.*, 1998, J Clin Invest, 102, 115-123). Briefly, on day 1, B6D2F1 mice received 1300 cGy total body irradiation (^{137}Cs source at 108 cGy/min), split into two doses separated by 3 h to minimise gastrointestinal toxicity. Donor bone marrow (5 x 10^6) and donor nylon wool purified splenic T cells (2 x 10^6) were resuspended in 0.25 ml of Leibovitz's L-15 media (Gibco BRL, Gaithersburg MD) and were injected intravenously into each recipient.

Survival was monitored daily, and GVHD clinical scores measured weekly. The degree of systemic GVHD was assessed by a scoring system which sums changes in five clinical parameters; weight loss, posture (hunching), activity, fur texture and skin integrity (maximum index = 10) (Hill *et al.*, 1997, Blood, 90, 3204-3213; Hill *et al.*, 1998, J Clin Invest, 102, 115-123; Cook *et al.*, 1996, Blood, 88, 3230-3239). Individual mice were ear-tagged and graded from 0 to 2 for each criterion without knowledge of treatment group. Animals with severe clinical GVHD (scores > 6) were sacrificed according to ethical guidelines and the day of death deemed to be the following day.

Inhibition of Cpn10 activity with anti-Hsp60 antiserum

RAW264-HIV-LTR-LUC cells were plated and incubated overnight as above. The medium was replaced and goat polyclonal anti-Hsp60 antiserum (1/1000 dilution) (Stressgen, Victoria, Canada), or the same dilution of normal goat serum was added for 1 hour. Cpn10 was then added for 2 hours, followed by LPS and luciferase activity measured 2 hours later.

Statistical Analysis

Statistical analysis was performed using univariate analysis of variance (ANOVA), t test or log rank statistic using SPSS for Windows 11.5.0 (SPSS Inc.).

Wound healing assay

C57 BL/6 mice were anesthetized with halothane and small surgical incisions (~ 0.5cm) were made in the skin between the scapulae under aseptic conditions. Osmotic pumps (ALZET 1007D) with perfusion rate of 0.5 ul/hr were filled with either Cpn10 (2.25 mg/ml) or control buffer. Using a haemostat, a

small pocket was formed by spreading connective tissue apart and osmotic pumps were implanted subcutaneously. The skin incisions were closed with sutures.

RESULTS

Inhibition of LPS signaling by Cpn10 using Raw-264-HIV-LTR-LUC indicator cells

To further investigate the role of Cpn10 as an immunosuppressive agent the ability of Cpn10 to inhibit LPS-mediated NF-κB activation was investigated. The RAW-264-HIV-LTR-LUC cells are a mouse macrophage cell line (RAW264.7) stably expressing a luciferase reporter gene with an HIV long terminal repeat promoter, which is highly and rapidly responsive to NF-κB stimulation. These cells provide a sensitive bioassay for analysis of TLR4 signaling pathways in macrophages stimulated with bacterial LPS (Sweet & Hume, 1995, J Inflamm, 45, 126-135). To avoid the use of supra-physiological levels of LPS, a titration range for LPS concentration was established, which represented approximately 80%, 50% and 20% of maximal LPS-stimulated luciferase activity (5, 1 and 0.2 ng, respectively) (data not shown). Preincubation of the reporter cells with 100 υg/ml of Cpn10 for 2 hours was able to inhibit significantly LPS-stimulated luciferase activity by 30-50% at these concentrations of LPS (Fig. 1). Shorter preincubation times provided less reproducible inhibition and preincubation times above 18 hours provided no inhibition (data not shown).

Cpn10-mediated inhibition of TNF-α and RANTES production in LPS-stimulated RAW264.7 cells

To illustrate that Cpn10-mediated inhibition of LPS-induced NF-κB activation translated to reduction in the secretion of pro-inflammatory mediators,

the ability of Cpn10 to inhibit LPS-induced production of the pro-inflammatory cytokine TNF-α and the pro-inflammatory chemokine RANTES was investigated. Incubation of Cpn10 with RAW264.7 cells significantly reduced LPS-induced TNF-α and RANTES secretion (Fig. 1B, C). Increasing the Cpn10 concentration did not significantly enhance the reduction of TNF-α secretion from these cells, and increasing the LPS concentration reduced the Cpn10-mediated reduction in TNF-α secretion (data not shown). The reduction in RANTES secretion induced by 5 ng/ml LPS was dose responsive for Cpn10 concentrations of 50-400 ug/ml; lower doses of LPS failed to induce readily detectable levels of RANTES (data not shown).

Cpn10-mediated inhibition of LPS-induced TNF-α was independent of IL-10

To determine the effect of Cpn10 in a more physiological cell population, mice were treated with Cpn10 and their peritoneal macrophages removed and stimulated with LPS *in vitro*. The Cpn10 treatment significantly reduced the LPS-induced secretion of TNF-α from these cells (Fig. 2A), illustrating that Cpn10 mediates similar effects on macrophages treated *in vivo* as seen with RAW264.7 cells treated *in vitro*.

IL-10 is a potent immunosuppressive cytokine able to inhibit TLR4 signaling (Berlato *et al.*, 2002, J Immunol, 168, 6404-6411; Suhrbier & Linn, 2003, Trends Immunol, 24, 165-168), and when splenocytes from Cpn10 treated animals were stimulated with LPS, significantly increased IL-10 production was observed compared to control animals (Fig. 2B). However, the Cpn10-mediated reduction in LPS-induced TNF-α production (Fig. 2A) did not require IL-10, since similar reductions in TNF-α secretion were observed when peritoneal

macrophages from Cpn10 treated IL-10$^{-/-}$ mice were stimulated with LPS *in vitro* (Fig. 2C). Thus, reduced TNF-α secretion and increased IL-10 production appear to be independent consequences of Cpn10 treatment.

Cpn10-treatment of human peripheral blood mononuclear cells (PBMC)

To determine whether Cpn10 is also active in human cells, PBMC from healthy donors were treated with a concentration range of LPS in the presence and absence of Cpn10. Cpn10-mediated an average 45-66% reduction in LPS-induced TNF-α secretion in a series of repeat experiments (Fig. 3A), illustrating that Cpn10 is also able to inhibit cytokine secretion from primary human PBMC. The ability of Cpn10 to inhibit LPS-induced TNF-α secretion was also shown to be dose dependent with increasing concentrations of Cpn10 mediating increasing inhibition (Fig. 3B). However, increasing levels of LPS reduced the ability of Cpn10 to mediate inhibition of LPS-induced TNF-α secretion (Fig. 3B). Cpn10 treatment also increased the level of LPS-induced IL-10 secretion in PBMC in a dose dependent fashion (Fig. 3C). In contrast to the Cpn10-mediated reduction in TNF-α secretion, Cpn10-mediated elevation of IL-10 production increased with increasing doses of LPS (Fig. 3C), again indicating that Cpn10 affects TNF-α and IL-10 independently.

Cpn10-treatment inhibited LPS-induced TNF-α secretion *in vivo*

A modified endotoxemia model was used to determine whether Cpn10 delivered *in vivo* was able to inhibit LPS-induced TNF-α secretion *in vivo*. BALB/c mice were given 100 υg of Cpn10 iv 30 mins before injection of 10 υg of LPS i.v., and blood removed after 1.5 hours. Cpn10 treatment resulted in an average 47.6% reduction in the serum TNF-α, an average 40.1% reduction in

serum RANTES, and an average 43.3% increase in serum IL-10 levels in several repeat experiments (Fig. 4A). Five days of daily Cpn10 pretreatment failed significantly to enhance this level of TNF-α inhibition (data not shown). These data are consistent with the previous tissue culture experiments and illustrate the *in vivo* efficacy of Cpn10 in reducing TNF-α and increased IL-10 production after challenge with LPS.

Cpn10 reduced the acute symptoms of Graft verses host disease (GVHD)

Acute GVHD following allogeneic bone marrow transplantation (BMT) is a T cell-mediated disease in which donor T cells recognise recipient allo-antigens and differentiate in a Th1 dominant fashion. The resulting T cell-derived Th1-cytokines (primarily IFN-γ) prime the donor mononuclear cells to release cytopathic quantities of inflammatory cytokines (e.g. TNF-α) when they are stimulated with LPS that has leaked through the radiation-damaged gastrointestinal mucosa. These cytokines and the allo-reactive T cells then contribute to increasing gastrointestinal damage and LPS leakage. GVHD mortality in BMT models is prevented if the donor mononuclear cells lack TLR4, LPS is effectively blocked (by therapeutic antagonists) (Cooke *et al.*, 2001, J Clin Invest, 107, 1581-1589), or TNF-α itself is neutralized. The ability of Cpn10 administration during the peri-transplant period to ameliorate GVHD was therefore investigated. Cpn10 treatment of transplant donors and recipients prior to transplant significantly delayed GVHD mortality (Fig. 4B). In addition, the severity of GVHD as determined by clinical score was also reduced early after BMT (Fig. 4B). Although Cpn10 was able to delay GVHD and reduce early morbidity, ultimately the animals succumbed to GVHD, consistent with the

inability of Cpn10 to abolish TNF-α secretion or to effect T cell proliferation and IFNγ secretion (Fig. 2). Treatment of animals with Cpn10 post transplant failed to affect significantly GVHD (data not shown).

Cpn10 and Hsp60 in TLR4 signaling

In the mitochondria Cpn10 binds to Hsp60 in the presence of ATP/ADP and magnesium ions. Both ATP and magnesium ions are also present in the extracellular fluids or medium (Beigi & Dubyak, 2000, J Immunol, 165, 7189-7198), and Hsp60 is believed to be present on the cell surface (Habich *et al.*, 2003, FEBS Lett, 533, 105-109; Shin *et al.*, 2003, J Biol Chem, 278, 7607-7616) where it contributes to TLR4 signaling. We therefore tested whether blocking Cpn10 binding to Hsp60 with anti-Hsp60 antiserum would prevent Cpn10-mediated inhibition of LPS-induced TLR4 signaling. Preincubation of RAW264-HIV-LTR-LUC cells with polyclonal anti-Hsp60 serum, but not control serum, prevented Cpn10-mediated reduction of LPS-induced cell activation, implicating cell surface Hsp60 as the target of Cpn10 activity.

Cpn10 and wound healing

The wounds of the mice were inspected 5 days after the procedure. In the group of animals treated with Cpn10 improved healing of the wounds was observed demonstrated by completely closed surgical incisions and the absence of surrounding inflammation in comparison to the buffer treated control mice.

Discussion

Cpn10 mediated a 25-70% inhibition of TNF-α secretion depending on the system used and the dose of LPS and Cpn10. Cpn10 increased LPS-induced IL-

10 secretion by approximately 30-200% depending on the system, but the inhibition of TNF-α secretion was not dependent on the elevation of IL-10.

Since *E. coli*-derived LPS is a well-described agonist for TLR4, the experiments indicate that Cpn10 modulates TLR4 signaling through the NF-κB pathway. However, it is likely that Cpn10 modulates cytokine secretion via other pathways stimulated by the TLR4 and TLR2 complex.

The inhibitory effects of Cpn10 are mediated very rapidly, within 30 mins (Fig. 4A) to 2 hours (Fig. 1A). This implicates inhibition of early signaling events or activation of rapid negative feedback mechanisms like PI3K, rather than late phase feedback mechanisms involving IRAK or SOCS (Fukao & Koyasu, 2003, *Trends Immunol,* 24, 358-363).

The specific PI3K inhibitor, wortmannin, had no detectable effect on Cpn10 activity (data not shown), suggesting Cpn10 does not affect the P13K pathway. Given the association of Hsp60 with TLR4 signaling and the known association of Cpn10 and Hsp60, we believe Cpn10 may inhibit LPS-induced TLR4 signaling through binding Hsp60. This notion is supported by the ability of anti-Hsp60 antibody to block Cpn10 activity (Fig. 5).

Hsp60 may be involved in augmenting LPS-induced TLR4 signaling (Johnson, *et al.,* 2003, Crit Rev Immunol, 23, 15-44), through incorporation into the TLR4 signaling complex/receptor (Triantafilou &Triantafilou, *supra*) and/or by binding to other activating receptors (Habich *et al., supra*; Barazi *et al.*, 2002, Cancer Res, 62, 1541-1548).

Through binding Hsp60, Cpn10 may prevent Hsp60 interaction with cell surface receptors and thereby inhibit Hsp60-mediated augmentation of TLR4 and

TLR2 signaling. Therefore it may be speculated that the physiological role of circulating Cpn10 during early pregnancy is to remove the Hsp60 danger signal produced during pregnancy.

Example 2 – Cpn10 and TLR2

Methods

BALB/C mice were injected subcutaneously with of ovalbumin (10 ug) (Sigma) emulsified in Complete Freund's adjuvant (CFA) (Sigma). CFA contains mycobacterial cell wall extracts which are believed to contain lipopeptide agonists of TLR2 (Lim *et al.*, 2003, Int Immunopharmacol, 3(1): 115-118; Tsuji *et al.*, 2000, Infect Immun; 68, 6883-6890; Kirschning & Schumann, 2002, Curr Top Microbiol Immunol, 270, 121-44). CFA is well known to induce granulomas (Bergeron *et al.*, 2001, Eur Respir J, 18(2), 357-361; Shah *et al.* 2001, J Assoc Physicians India, 49, 366-368).

Cpn10 (100 ug) was given twice daily for 5 days with two doses preceding injection of the CFA. Subcutaneous granulomas were measured at the indicated times.

Results

To determine whether Cpn10 is able to infect CFA's granuloma formation activity, Cpn10 treated and buffer control treated mice where injected with CFA. Cpn10 treatment significantly reduced the size of the granuloma induction (Fig. 6A).

Discussion

As CFA stimulates TLR2 and CFA induces granuloma formation, a reduction in granuloma formation mediated by Cpn10 treatment provides evidence that Cpn10 also inhibits TLR2 signaling.

Cpn10 inhibits activation of NF-κB by the TLR2 agonist $PAM_3CYS\text{-}SK_4$

$PAM3CysSK_4$ (a lipopeptide) is a known agonist of TLR2 (Agrawal *et al.*, J Immunol, 2003, 171, 4984-9) and is able to stimulate the HIV LTR (Fig. 6B) which is thought to activate transcription factors, such as NF-κB (Lee *et al.*, J Immunol. 2002. 168(8):4012-4017).

Material and Methods

PAM_3CysSK_4 was purchased from EMC Microcollection GmbH and dissolved as a working stock dilution of 1 mg/ml in water. Cpn10 and the RAW 264 -HIV LTR Luc assay system was performed as described previously for LPS. Briefly Raw-luc cells were seeded at 2.5×10^5 cell/ml into 24 well plates and incubated overnight at 37 C. Cpn10 was added to the cells at 120 ug/ml and incubated for 2h at 37^0C. $PAM_3Cys\text{-}SK_4$ (10 ng/ml or 2 ng/ml or 0 ng/ml) was then added for 2 h prior to the luiferase assay. LPS at 1 ng/ml or 0.2 ng/ml was used as a positive control.

Results

Cpn10 inhibited HIV LTR activation by the TLR2 agonist $PAM3CysSK_4$ (Fig. 6B). This inhibition was maintained irrespective of whether the medium was changed before addition of Cpn10 (Fig. 6C) or before addition of $PAM3CysSK_4$ (Fig. 6D).

Conclusion

Cpn10 is able to inhibit pro-inflammatory mediator activation signals in macrophages stimulated by a TLR2 agonist.

Example 3 – Cpn10 and TLR3

Methods

RAW264-HIV-LTR-LUC cells were stimulated with poly IC (synthetic double stranded RNA) using the same methods described above for LPS stimulation. Double stranded RNA is an agonist of TLR3 and stimulates NF-κB (Suhrbier & Linn, 2003, Trends Immunol, 24(4), 165-168).

Results

To determine whether Cpn10 was able to inhibit polyIC-induced TLR3 signaling, RAW264-HIV-LTR-LUC cells were treated with 100 ug/ml of Cpn10 for 2 h followed by polyIC for 2 h before analysis of the LUC (luciferase) levels was undertaken. The % inhibition of the RLU (relative light unit) is shown (Fig. 7). As a positive control inhibition of LPS/TLR4 inhibition was conducted in parallel control.

Discussion

As polyIC is a known TLR3 agonist these data indicate that Cpn10 is able to inhibit TLR3 signaling.

Example 4 – Cpn10 and cachexia

Methods

Induction of Adjuvant-induced cachexia

The aim of the present study was to determine whether Cpn10 administration to rats during development of adjuvant arthritis would result in decreased weight loss. Female Dark Agouti rats (n=30, 150-160 g) were injected

subcutaneously with 0.1 ml of Complete Freund's adjuvant (CFA) at the base of the tail. The adjuvant consisted of incomplete Freund's adjuvant (Difco, Michigan, USA) to which was added 10 mg/ml heat-killed *Mycobacterium tuberculosis* H37RA (Difco). The onset of detectable arthritic disease in this model is generally 8-10 days after CFA injection.

Cpn10 Treatment

Rats were injected subcutaneously with 0.25 mg/kg (n=10) or 2.5 mg/kg Cpn10 (n=10) or diluent control (Tris/saline buffer) (n=10) daily from day 2 to day 13, and weighed on a daily basis.

Statistical Analysis

The difference in weight observed in rats receiving Cpn10 or diluent control were tested for significance using univariate analysis of variance (ANOVA).

Results

There was a net loss of weight in all groups following administration of CFA, which appeared more marked in the control group (Fig. 8).

When the weight loss data for the 0.25 and 2.5 mg/kg Cpn10 treatment groups were pooled and compared with the control group, there was a statistically significant difference (p=0.027) in weight loss. The pooling of data from the two treatment groups is justified in this case due to the similar values of weight loss in the two groups (p=0.94) over this time period.

Discussion

Adjuvant arthritis leads to changes in body composition and cytokine production that mimics pro-inflammatory cytokine-driven cachexia in chronic

inflammatory arthritis (Mayer, 1997, Arthritis Rheum, 40, 534-539). Cpn10 administration *in vivo* or *in vitro* reduces production of pro-inflammatory cytokines by cells stimulated by LPS and other agonists.

The effects of Cpn10 were tested at two doses in rats in which cachexia was induced experimentally with a single injection of CFA. Diluent control and Cpn10 were administered subcutaneously to animals of similar weight and age. The administration of CFA resulted in a significant decrease in body weight. By comparing the Cpn10 treated versus control treated groups, there was a statistically significant reduction in weight loss in the Cpn10-treated rats.

Elevated levels of inflammatory cytokines including TNF-α, IL-1β and IL-6 are known to correlate with cachexia in a number of diseases, including cancer and rheumatoid arthritis (Argiles, 2003. Curr Opin Clin Nutr Metab Care, 6(4); 401-406; Walsmith, 2002, Int J Cardiol, 85, 89-99). We have shown that administration of Cpn10 reduces production of TNF-α and RANTES, and increases production of the anti-inflammatory cytokine IL-10 in murine models of endotoxemia and graft-versus-host-disease, and *in vitro* LPS-stimulation of freshly isolated PBMC and monocyte cell lines.

Example 5 – Effect of Cpn10 in human clinical trial subjects

Methods

Phase I Clinical Trial

Twenty healthy normal volunteers aged between 18 and 55 years were enrolled in a 14-day phase I trial of Cpn10 to assess the pharmacokinetics and safety of Cpn10 administered as a single intravenous infusion or subcutaneous injection in a double-blind placebo control protocol. Following screening and

written informed consent, subjects were fasted overnight prior to dosing with Cpn10 at 1, 2.5, 5 or 10 mg given as a 10 minute intravenous infusion, or 5 mg given subcutaneously. Blood samples (50 ml) for PBMC isolation were collected prior to dose (approximately 12 hours pre-dose), 8 hours post-dose, and on day 6 following Cpn10 dose. Subjects were monitored for 14 days post treatment for adverse events, with blood drawn at intervals for standard haematology and biochemistry assessment and development of anti-Cpn10 antibodies.

PBMC isolation and storage

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by density gradient centrifugation on Ficoll-Hypaque Plus (Amersham) using the manufacturer's protocol. Following two wash steps, cells were resuspended in freezing medium (10% DMSO in foetal bovine serum [FBS]) and frozen using a step-down freezing method at –80°C. Cells were transported to Cbio on dry ice and then stored in liquid nitrogen until use.

PBMC stimulation

PBMC from cohorts 1, 2 and 3, day 0 and day 1 were thawed and centrifuged through FBS followed by washing and resuspending in RPMI with 10% FBS. Cells were aliquotted at a final density of 1 x 10^6 viable cells/ml in 24-well tissue culture plates with or without LPS. Following 20 hrs incubation at 37°C with 5% CO_2, cell culture supernates were collected and tested for levels of TNF-α using a commercial human TNF-α ELISA (R&D Systems).

Results

Figure 9 A and B demonstrate a dose-responsive Cpn10-mediated change in TNF-α production by PBMC stimulated with a range of LPS concentrations *in*

vitro. The LPS-driven response at day 1 vs. day 0 (i.e. post-Cpn10 vs. pre-Cpn10) was compared in groups of volunteers given 1 (n=1), 2.5 (n=3) or 5 mg Cpn10 (n=3) or placebo (n=3).

Discussion

As a predictor of immune activity in humans during a phase I clinical trial, peripheral blood mononuclear cells (PBMC) were collected before, and 8 hours after a single intravenous infusion of Cpn10. The cells were stimulated with a range of LPS concentrations *in vitro* in the absence of exogenous Cpn10. The data support the hypothesis that Cpn10 administered as a single intravenous dose to humans *in vivo*, reduces the pro-inflammatory cytokine response following LPS-stimulation *ex vivo* in a dose-responsive manner.

The PBMCs were isolated at a time-point (8 hrs post dose) at which the Cpn10 in the serum could no longer be measured, supporting a view that while the serum half-life of this recombinant protein is short (approximately 1 hr), its biological effects may be longer-lived.

Throughout the specification the aim has been to describe the preferred embodiments of the invention without limiting the invention to any one embodiment or specific collection of features. It will therefore be appreciated by those of skill in the art that, in light of the instant disclosure, various modifications and changes can be made in the particular embodiments exemplified without departing from the scope of the present invention.

All computer programs, algorithms, patent and scientific literature referred to herein is incorporated herein by reference.

DATED this sixteenth day of January 2004

CBIO LIMITED

by its Patent Attorneys

FISHER ADAMS KELLY

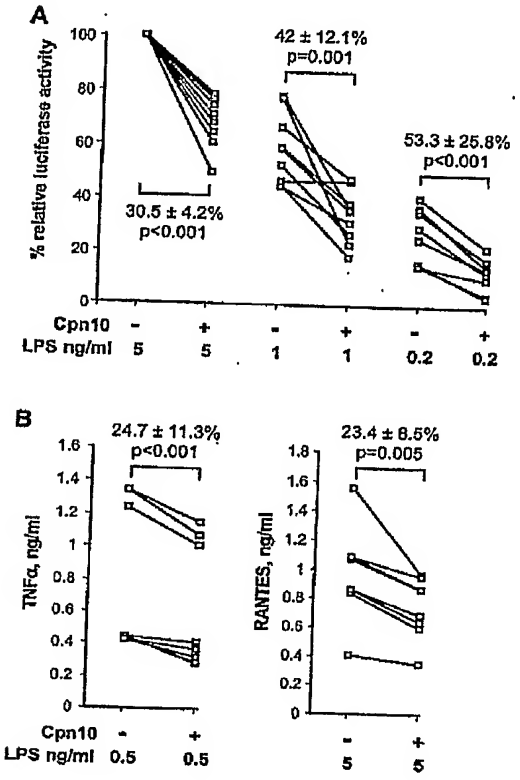
A
% relative luciferase activity
100
80
60
40
20
0
30.5 ± 4.2%
p<0.001
42 ± 12.1%
p=0.001
53.3 ± 25.8%
p<0.001
Cpn10 - + - + - +
LPS ng/ml 5 5 1 1 0.2 0.2
B
TNFα, ng/ml
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
24.7 ± 11.3%
p<0.001
Cpn10 - +
LPS ng/ml 0.5 0.5
RANTES, ng/ml
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
23.4 ± 8.5%
p=0.005
- +
5 5

FIG. 1

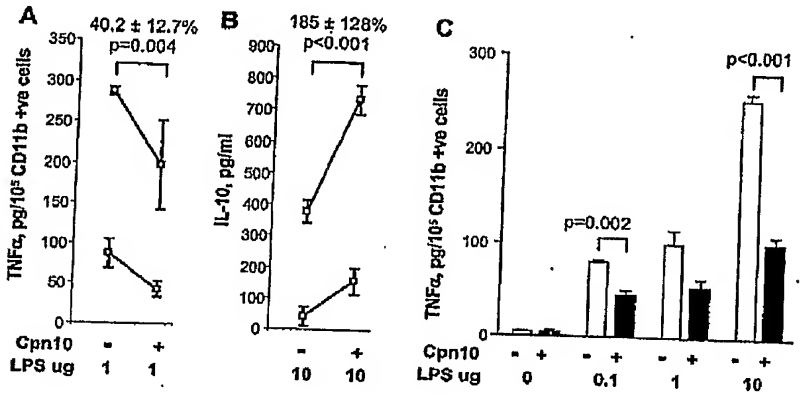
A
40.2 ± 12.7%
p=0.004
TNFα, pg/10⁵ CD11b +ve cells
350
300
250
200
150
100
50
0
Cpn10 - +
LPS ug 1 1
B
185 ± 128%
p<0.001
IL-10, pg/ml
900
800
700
600
500
400
300
200
100
0
- +
10 10
C
TNFα, pg/10⁵ CD11b +ve cells
300
200
100
0
p=0.002
p<0.001
Cpn10 - + - + - + - +
LPS ug 0 0.1 1 10

FIG. 2

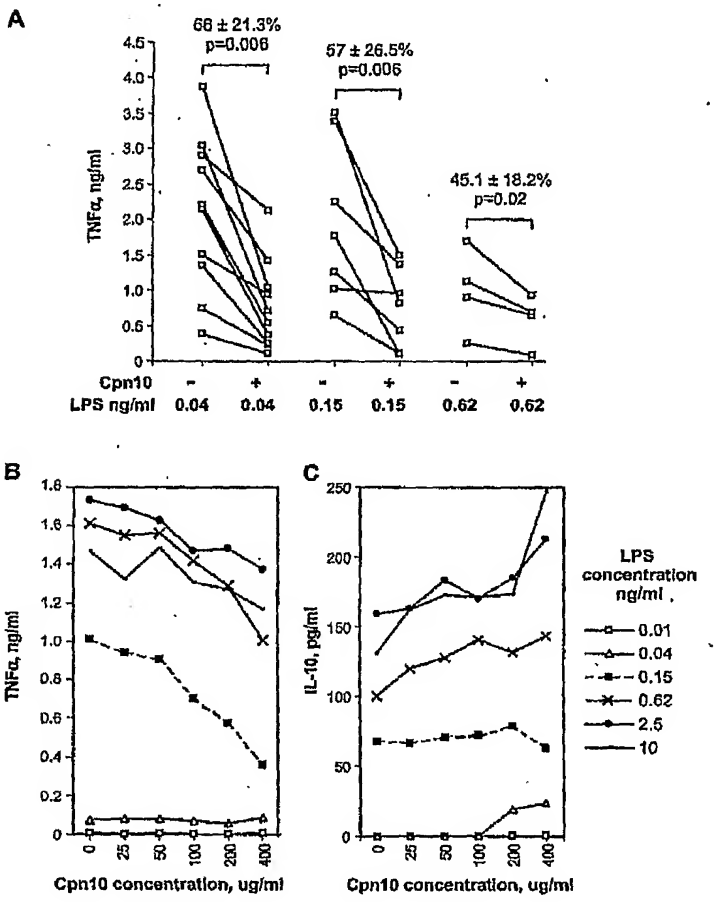
A
68 ± 21.3%
p=0.006
57 ± 26.5%
p=0.006
45.1 ± 18.2%
p=0.02
TNFα, ng/ml
Cpn10
LPS ng/ml
0.04
0.15
0.62
B
TNFα, ng/ml
Cpn10 concentration, ug/ml
C
IL-10, pg/ml
Cpn10 concentration, ug/ml
LPS concentration ng/ml
0.01
0.04
0.15
0.62
2.5
10

FIG. 3

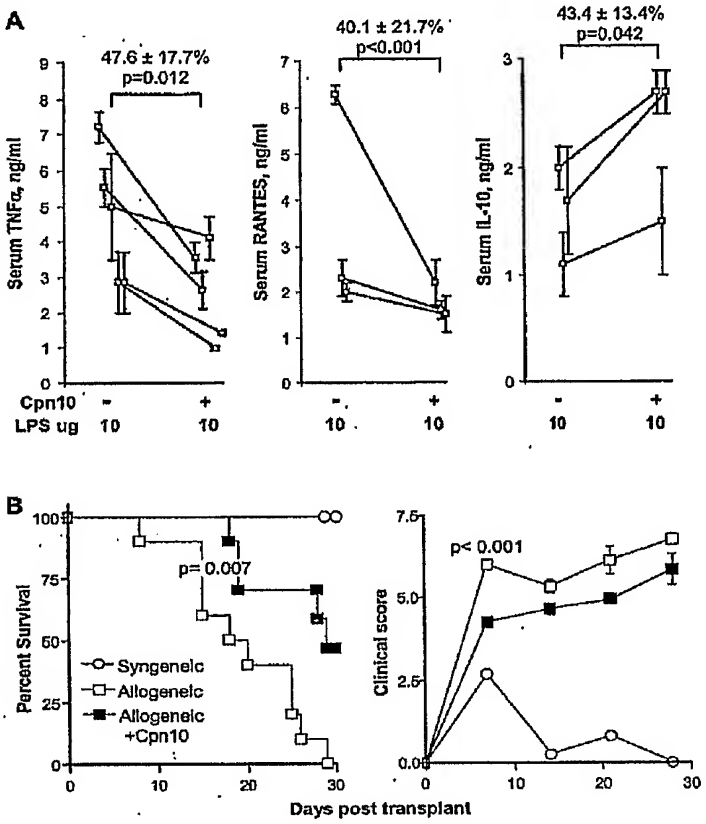
A
47.6 ± 17.7%
p=0.012
40.1 ± 21.7%
p<0.001
43.4 ± 13.4%
p=0.042
Serum TNFα, ng/ml
Serum RANTES, ng/ml
Serum IL-10, ng/ml
Cpn10 - + - + - +
LPS ug 10 10 10 10 10 10
B
Percent Survival
p= 0.007
Syngeneic
Allogeneic
Allogeneic +Cpn10
Clinical score
p< 0.001
Days post transplant

FIG. 4

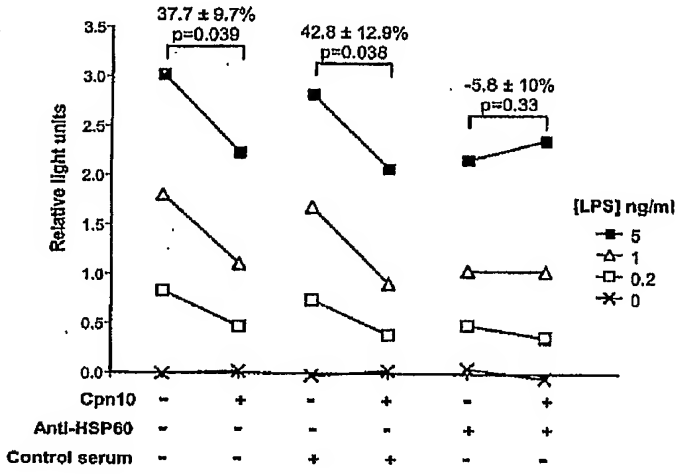
37.7 ± 9.7%
p=0.039
42.8 ± 12.9%
p=0.038
-5.8 ± 10%
p=0.33
Relative light units
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
[LPS] ng/ml
5
1
0.2
0
Cpn10 - + - + - +
Anti-HSP60 - - - - + +
Control serum - - + + - -

FIG. 5

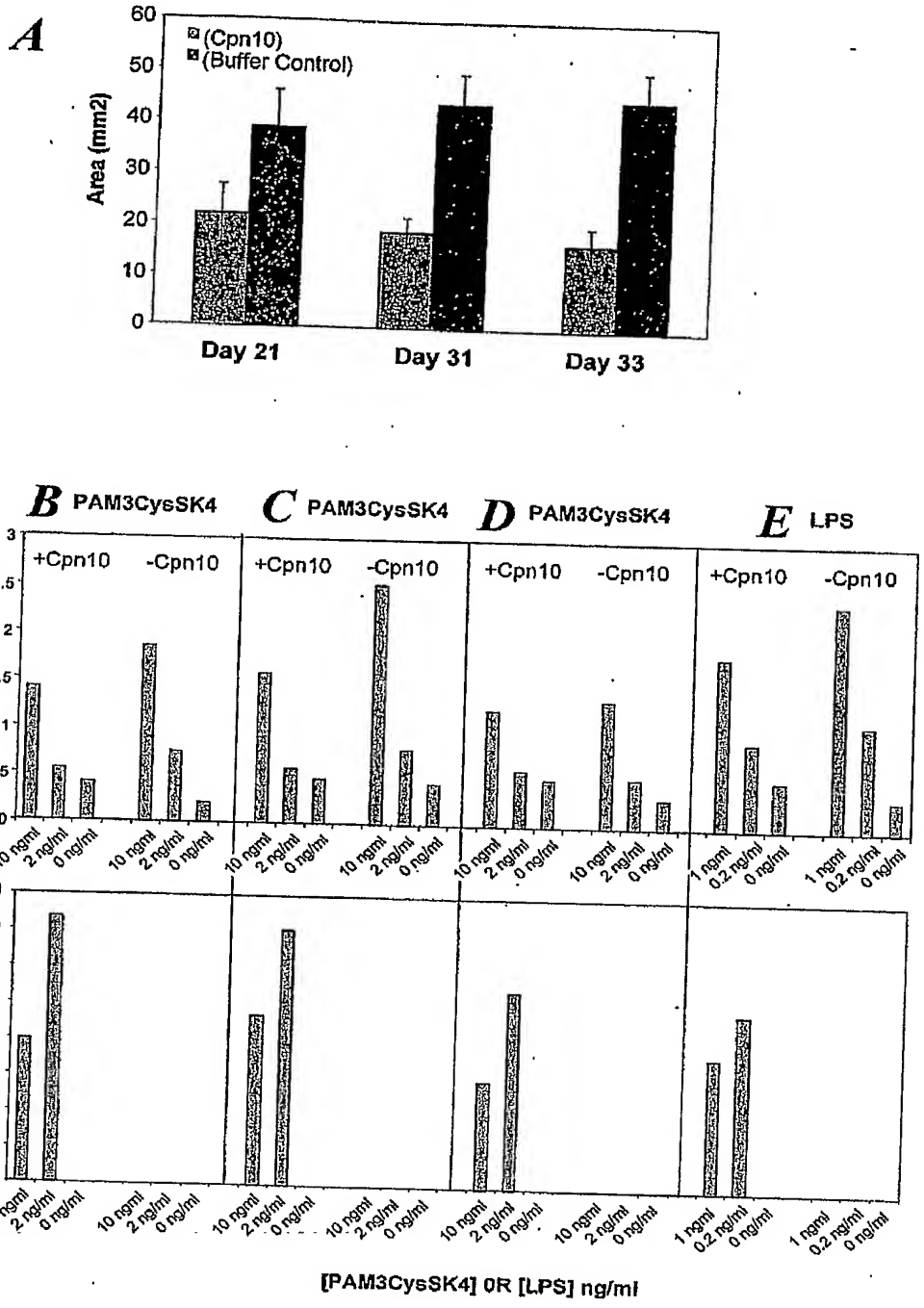
A
(Cpn10)
(Buffer Control)
Area (mm2)
Day 21
Day 31
Day 33
B PAM3CysSK4
C PAM3CysSK4
D PAM3CysSK4
E LPS
+Cpn10
-Cpn10
RLU
%INHIBITION
10 ngml
2 ng/ml
0 ng/ml
1 ngml
0.2 ng/ml
[PAM3CysSK4] 0R [LPS] ng/ml

FIG. 6

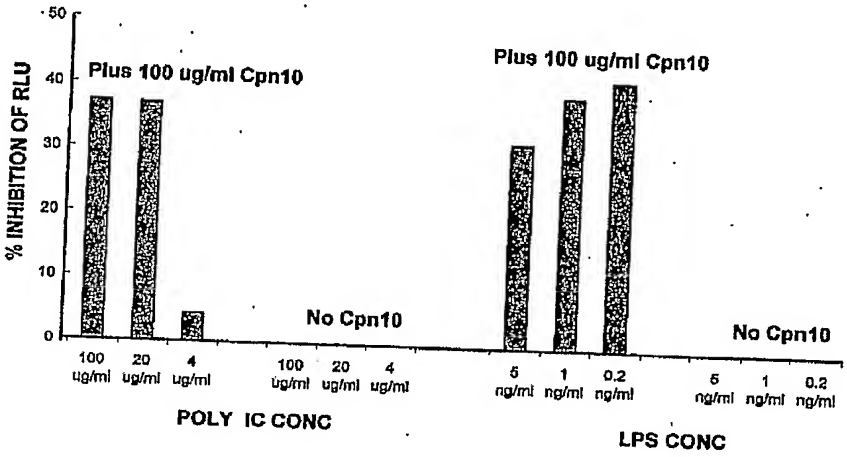
% INHIBITION OF RLU
50
40
30
20
10
0
Plus 100 ug/ml Cpn10
No Cpn10
100 ug/ml
20 ug/ml
4 ug/ml
100 ug/ml
20 ug/ml
4 ug/ml
POLY IC CONC
Plus 100 ug/ml Cpn10
No Cpn10
5 ng/ml
1 ng/ml
0.2 ng/ml
5 ng/ml
1 ng/ml
0.2 ng/ml
LPS CONC

FIG. 7

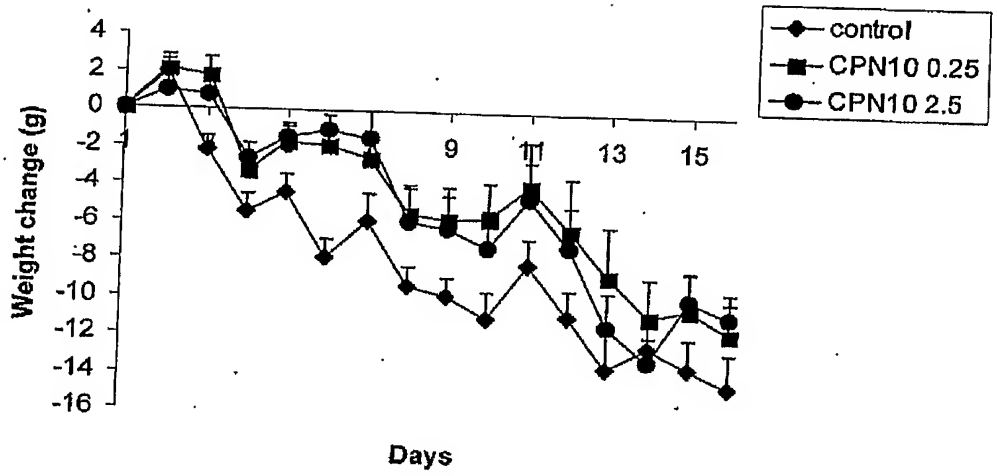
control
CPN10 0.25
CPN10 2.5
Weight change (g)
4
2
0
-2
-4
-6
-8
-10
-12
-14
-16
9
11
13
15
Days

FIG. 8

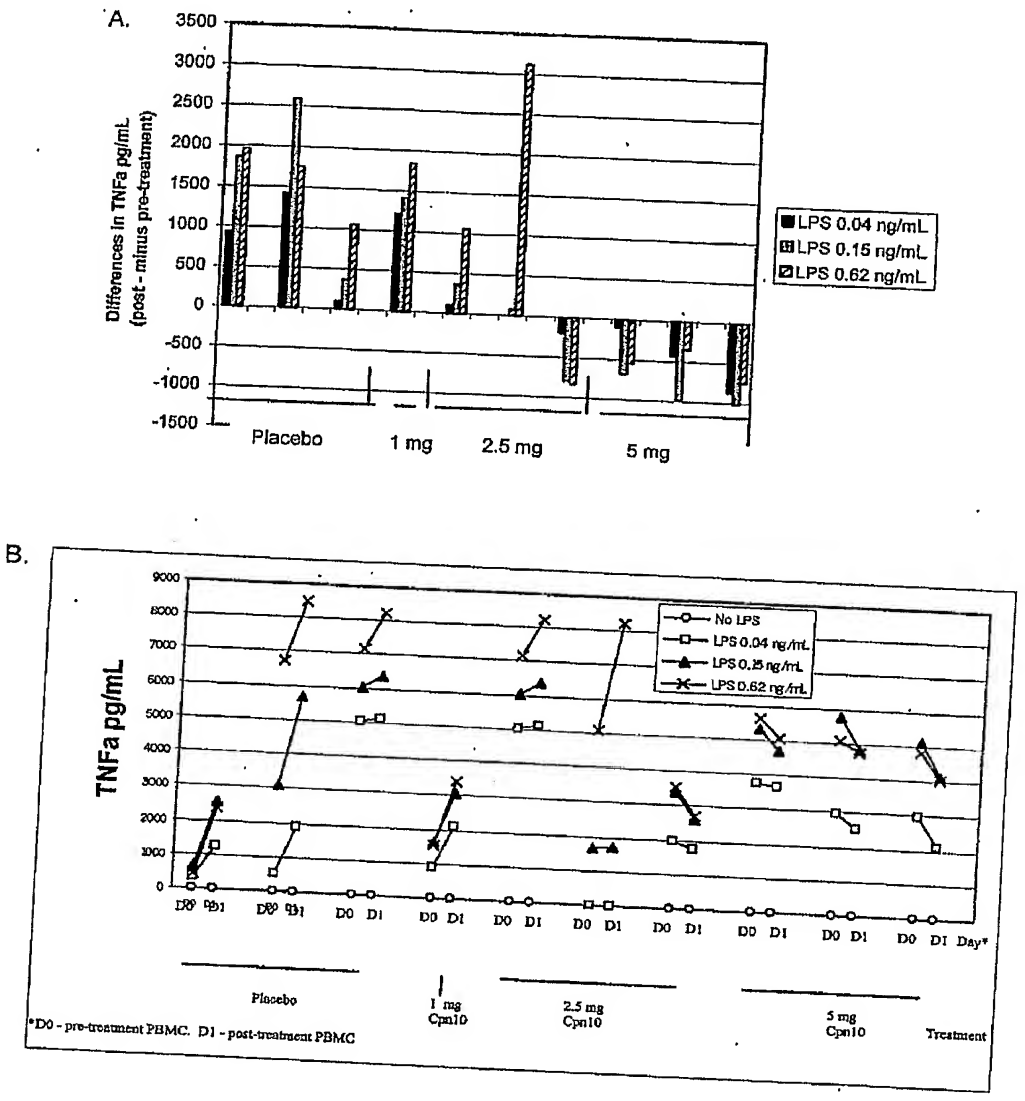
A.
Differences in TNFa pg/mL (post - minus pre-treatment)
3500
3000
2500
2000
1500
1000
500
0
-500
-1000
-1500
Placebo
1 mg
2.5 mg
5 mg
LPS 0.04 ng/mL
LPS 0.15 ng/mL
LPS 0.62 ng/mL
B.
TNFa pg/mL
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
No LPS
LPS 0.04 ng/mL
LPS 0.25 ng/mL
LPS 0.62 ng/mL
D0 D1
Day*
Placebo
1 mg Cpn10
2.5 mg Cpn10
5 mg Cpn10
Treatment
*D0 - pre-treatment PBMC. D1 - post-treatment PBMC

FIG. 9